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14. ABSTRACT
Genomic alterations of 18q have been observed in prostate cancer. This research focuses on analyzing the role of increased gene copy number at 18q22.1 in prostate cancer. We believe the key genes in this region are type II cadherins. We are studying the role of overexpression of cadherin 7 (CDH7) on the tumorigenic and invasive potential of prostate cancer cells. The increased copy number of CDH7 is specific to prostate cancer and results in increased levels of CDH7 mRNA in prostate tumors. We previously created and purifiedpolyclonal antibodies against CDH7 peptides to use in immunohistochemistry experiments to determine if increased CDH7 copies increases the protein level, but these antibodies proved to be inadequate. We have since created polyclonal and monoclonal antibodies against full-length bacterially-expressed protein and are in the process of testing these antibodies. We have already performed knockdown experiments of the CDH7 mRNA in a prostate cancer cell line and will analyze the CDH7 protein levels in these cells with these new antibodies. We will subsequently evaluate these knockdown cells for invasive and tumorigenic potential.

15. SUBJECT TERMS
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Introduction

Genomic alterations at 18q have been observed in prostate cancer. This research focuses on analyzing the role of copy number increase at 18q22.1 in prostate cancer and the potential function of the critical genes that are found to be present in increased copy numbers. This is innovative research in that we are the first to observe increased copy numbers of genes in this region in prostate cancer. We believe the key genes in increased copy are a class of cell adhesion molecules, the type II mesenchymal cadherins. We are studying the role of overexpression of these genes normally expressed in mesenchymal cells, particularly cadherin 7 (*CDH7*), on the tumorigenic and invasive potential of prostate cancer epithelial cells. These cadherins have never been implicated in prostate cancer, despite the fact that the CDH7 protein is only expressed in brain, testes and prostate. We will analyze the expression of a variety of cadherin gene family members in prostate tumors of varying stages and grades. The results of the study will be able to demonstrate whether overexpression of these mesenchymal-type adhesion molecules is a predisposing factor for prostate cancer development.

Body

The research accomplishments for:

Task 1: Identify the smallest common region of amplification at 18q22.1 in prostate cancer.

a. Perform fluorescence in situ hybridization (FISH) on paraffin-embedded prostate tumor specimens using bacterial artificial chromosomes (BACs) spanning the amplified region.

We previously assembled a complete contig of BAC clones that spanned the original region of altered copy number defined by array comparative genomic hybridization (array CGH). Using FISH methodologies on prostate tumors with hybridization probes created by fluorescently-labeling DNA isolated from two BAC clones that flank the *CDH7* gene, we have shown that the smallest region of altered copy number is a 680 kb region, a portion of which is homologous to the chromosome 18 genome found in the BAC clone RPCI11-775G23, which encodes *CDH7* (Figure 1).

a. **b.**

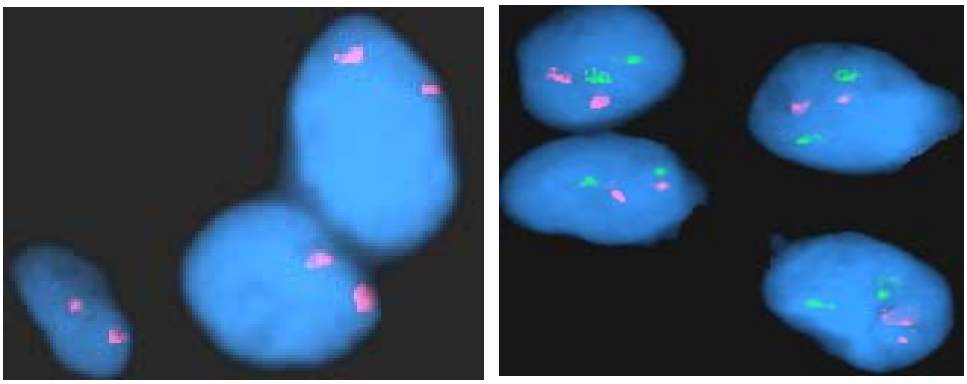

Figure 1: Identification of minimal region of amplification at 18q22.1. A probe proximal to RPCI11-775G23, RPCI11-453M23 (Spectrum Orange) is present in two copies in both tumor (a) and normal prostate tissues (b), while a distal probe RPCI11-425M2 (FITC) is deleted in the prostate tumor section (a). These two probes are present approximately 680 kb apart from each other on the chromosome.

b. Perform quantitative PCR on genomic DNA isolated from prostate tumor specimens.

Since the *CDH7* gene is located within this region of increased copy number, we analyzed the copy number of *CDH7* using quantitative PCR. DNA was isolated from microdissected prostate tumors showing increased copy number at 18q22.1 and a quantitative assay to measure *CDH7* gene copy number using real-time PCR was developed. The copy number of the *CDH7* gene in the prostate tumors ranged between two and seven (data not shown). For the majority of samples, the gene copy number of *CDH7,* as detected by quantitative PCR, correlated with the degree of amplification of the region homologous to RP11-775G23 detected by array CGH in the corresponding tumor section.

c. Perform quantitative reverse transcription-PCR on RNA isolated from prostate tumors to verify increased gene expression with increased gene copy number.

We tested RNA extracted from microdissected prostate tumors to verify whether the genomic amplification of the gene has any impact at the transcriptional level of *CDH7*. As expected, we detected three- to eight-fold overexpression of *CDH7* mRNA in prostate tumors compared to the normal adjacent tissue (Figure. 2). The cell line PZ-HPV-7, derived from human prostate epithelial cells, showed very low *CDH7* expression. The expression of *CDH7* was several hundred-fold higher in the tumors, compared to PZ-HPV-7 (Fig. 2). Together, array CGH, FISH and real-time quantitative PCR show increased copy number of the *CDH7* gene in prostate cancer which correlates with increased expression of *CDH7*.

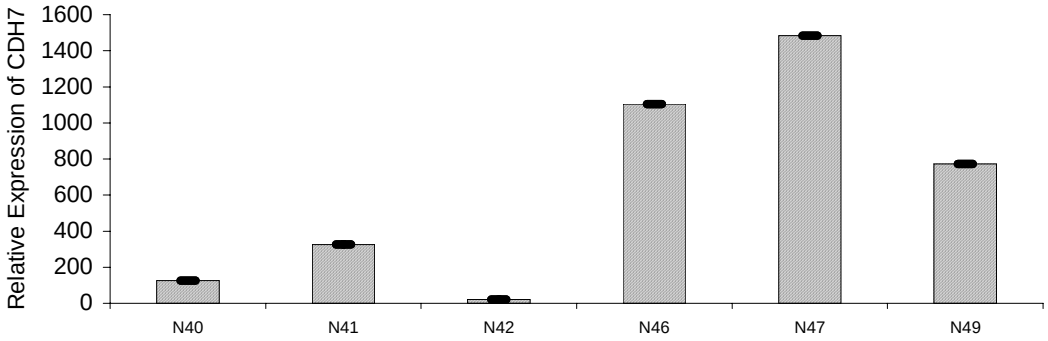

Figure 2: Quantitative real-time RT-PCR analysis of *CDH7* mRNA in prostate tumors. The human prostate epithelial cell line PZ-HPV-7 was given a value of 1 for *CDH7* expression and the prostate tumors were shown as fold expression above the level of PZ-HPV-1

Task 1 is completed.

Task 2: Investigate the expression of E-cadherin, N-cadherin, cadherin-7, cadherin-11, cadherin-19 and cadherin-20 proteins in prostate tumors of varying stages and grades.

We are investigating the expression of E-cadherin, N-cadherin and cadherin-11 in prostate tumors because E-cadherin has been found to be down-regulated in prostate tumors (Rubin et al., 2001) and co-expression of two mesenchymal cadherins N-cadherin and cadherin-11 have been reported in prostate cancer samples (Tomita et al., 2000). *CDH7* is within our minimal region of increased copy number and the cadherin-19 gene is approximately 700 kb distal to the

CDH7. The gene for another mesenchymal cadherin, cadherin-20 is located 5 megabases proximal to *CDH7* and does not appear to be in increased copy number, but its expression in prostate cancer needs to be assessed. This study will give a more complete picture of changes in cadherin expression during prostate cancer progression.

a. Create antibodies to specifically recognize cadherin-7 and -19.

As reported in our first annual report (November 2006), we contracted with Sigma-Genosys to create two custom polyclonal antibodies against CDH7 and CDH19 using unique peptides from the extracellular domain and the COOH-terminus. We received the antisera and performed some preliminary characterization. Our initial results showed that the antibodies directed against the extracellular domain peptides appeared to recognize the appropriate size of protein from cell lysates, as detected by western blotting. However, prior to their use in immunohistochemistry experiments, the antisera needed further purification. We attempted many types of purification strategies, and a collaborator had success with the antibodies on paraffin-embedded brain tissue, but the antibodies directed against the small peptides proved to be inadequate for immunohistochemical analysis of paraffin-embedded prostate tissue.

Since the peptide antibodies were not suitable for our purposes, we have created rabbit polyclonal and mouse monoclonal antibodies directed against bacterially-expressed full-length CDH7 and CDH19 with the help of the UTHSCSA Institutional Antibody core laboratory. We have preliminary information demonstrating that we have mice showing strong reactivity to CDH7 and CDH19 antigens and low reactivity to either GST or MBP alone (Figure 3). These mice were used to create hybridoma cell lines and we are in the process of testing these monoclonal supernatants by western analysis and on paraffin-embedded-prostate sections.

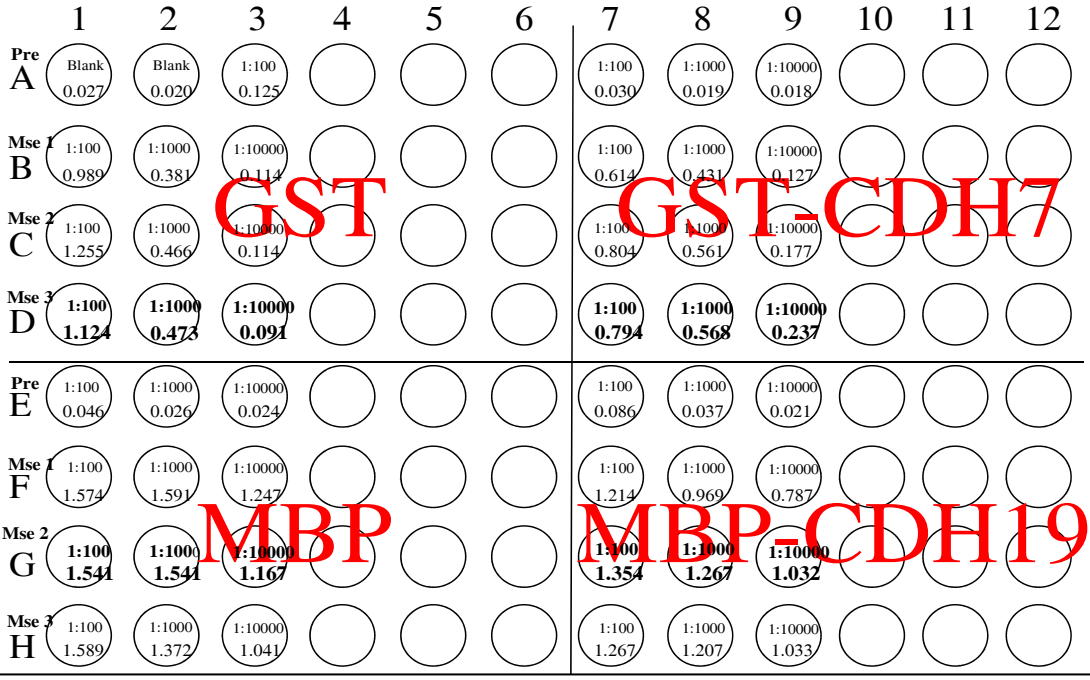

Figure 3: ELISA assay using CDH7 and CDH19 antigens. Top Panel. Mouse 3 serum showed good reactivity to CDH7 and low reactivity to GST. Bottom panel. Mouse 2 serum showed moderate reactivity to CDH19 and low reactivity to MBP.

b. Prepare tissue microarrays using prostate cancer specimens of various stages and grades.

Imgenex (San Diego, CA) is a commercial supplier of tissue microarrays and they have developed a prostate tumor microarray consisting of 40 tumors of various stages and Gleason grades with matched normal tissue. FISH was performed on this microarray using a hybridization probe derived from the BAC clone RPCI11-775G23 that contains the *CDH7* gene. All 40 prostate tumors showed three or more signals from the RPCI11-775G23 probe (data not shown). The control probe, a centromeric probe from chromosome 18, showed one to two signals in all 40 tumors on the array indicating that chromosome 18 was not completely amplified in the tumors. These results were consistent with our original array CGH data which showed no correlation between *CDH7* copy number and Gleason score (Table 1). This leads us to hypothesize that the mechanism that results in increased copies of *CDH7* is an early event during tumorigenesis and may be a predisposing factor to the development of prostate cancer.

Table 1. Prostate tumor samples with various pathological grades showing amplification at chromosome 18q22.1

Tumor	Age	Gleason	Stage	Amplification at 18q22.1
N10	66	6	T2CNXMX	Medium*
N12	64	6	T2CNXMX	Medium
N15	72	7	T2CNXMX	Medium
N22	59	7	T2CN0MX	High
N26	56	6	T2CNXMX	Low
N30	73	5	T2CNXMX	Medium
N31	72	6	T2CNXMX	Medium
N32	54	8	T2CN0MX	Low
N34	52	7	T3BN0MX	Normal
N35	56	7	T2CNXMX	Low
N36	62	6	T2CNXMX	Medium
N37	62	9	T3AN0MX	High
N38	59	6	T2CNXMX	Medium
N39	60	7	T3NXMX	Low
N40	70	7	T2CNXMX	High
N41	67	7	T3BN0MX	High
N42	67	8	T2CN0MX	Low
N43	65	5	T2BNXMX	Low
N44	53	7	T2BNXMX	Medium
N45	55	9	T2BN0MX	Low
N47	71	9	T3BN0MX	Low
N49	56	9	T2CNXMX	Low

* Log_2 ratio 1-1.5: Low; Log_2 ratio 1.5-2.0: Medium; Log_2 ratio >2.0: High

c. Perform immunohistochemistry experiments to analyze expression of E-cadherin, N-cadherin and the type II cadherins, cadherin-7, -11, -19 and -20 using tissue microarrays.

Since we have had great difficulty with our development of antibodies against CDH7 and CDH19 which will work for immunohistochemistry experiments, we have not been able to proceed with this task. We have great hope that our new monoclonal antibodies will allow us to analyze paraffin-embedded tissue sections for CDH7 protein levels. Antibodies to E-cadherin, N-cadherin and cadherin-11 are commercially-available and the amount of protein of these cadherins will also be analyzed to provide a more complete picture of the cadherin expression changes occurring during the tumorigenic process in prostate.

d. Perform immunohistochemistry experiments to test the tissue specificity of the three type II cadherins (cadherin-7, -19 and –20).

We performed FISH experiments with the RPCI11-775G23 (contains *CDH7*) probe on tissue microarrays consisting of cancer from 12 organ sites (Imgenex common cancers A and B) The gene copy number alteration detected at 18q22.1 using probe RPCI11-775G23 on prostate tumor samples was not observed in any other tumors samples, including stomach, esophagus, lung, colon, thyroid, kidney, breast, liver, urinary bladder, ovary and pancreas. These data indicate that the increased copy number of the RPCI11-775G23 region containing *CDH7* is tumor specific, and is limited to the prostate. Of these 12 tissues analyzed, only prostate is known to express *CDH7* (Kools et al., 2000). Since *CDH7* is also expressed in testes (Kools et al., 2000), we have analyzed a small number of testicular tumors by FISH and have shown that *CDH7* is also in increased copy number. We are in the process of expanding this observation.

Task 3: Knockdown expression of type II cadherins in prostate cancer cell lines and analyze the phenotype of the cells for invasive and tumorigenic potential.

a. Create transient and stable RNA interference constructs and perform experiments to knockdown expression of cadherin-7, -19 or -20, individually.

We designed short hairpin RNAs (shRNA) for cadherin-7, -19 and -20 using design tools provided by Ambion (The Woodlands, TX). The shRNA was cloned into the pSilencer vector (Ambion) which contains a mammalian selectable marker for creating RNA interference constructs that can be stably selected in a mammalian cell line. The shRNA construct for *CDH7* was stably transfected into the 22Rv1 human prostate cancer cell line, a cell line that expresses *CDH7*. Analysis of the *CDH7* mRNA in the stable transfectants showed dramatic reduction in the mRNA levels, but these results need to be confirmed by performing western blots using our newly-developed monoclonal antibodies,to determine the levels of CDH7 protein.

b. Analyze the transformed metastatic phenotype of the prostate cancer cells after knockdown of the type II cadherins using *in vitro* assays for anchorage independence and migration/invasion.

c. Analyze the *in vivo* tumorigenic phenotype of the prostate cancer cells after knockdown of type II cadherin mRNA.

Due to our problems in creating antibodies, no work to date has been accomplished on Tasks 3b or 3c. These experiments will be performed after we have shown a successful knockdown of the cadherin protein in the 22Rv1 cells stably transfected with shRNA, using the antibodies we

are testing from Task 2a. We have experience in both the *in vitro* anchorage independence and migration assays and in performing the in vivo analysis of tumorigenic phenotype, so these experiments will be completed quickly once we have determined the appropriate cells to test.

Key Research Accomplishments (To Date)

- **Defined a minimal region of increased copy number (680kb) at 18q22.1 containing the cadherin 7 gene in prostate cancer.**
- **Determined that increased copy number of the cadherin-7 gene results in increased cadherin-7 mRNA levels in prostate tumors**
- **Determined that increased copy numbers of the cadherin-7 gene do not correlate with the stage or Gleason score of prostate tumors.**
- **Determined that increased copy number of the cadherin-7 gene is found specifically in prostate cancer and not 11 other common cancers.**
- **Determined that increased copies of the cadherin-7 gene are also found in testicular cancer**
- **Created rabbit polyclonal antibodies and mouse monoclonal antibodies to full-length cadherin-7 and -19 proteins.**
- **Knocked down expression of the cadherin-7 gene in the prostate cancer cell line 22Rv1.**

Reportable Outcomes

We have submitted our work as a manuscript to the scientific journals Genes, Chromosomes and Cancer and Oncogene, however without data demonstrating the amount of CDH7 protein in the prostate tumors our manuscript has not been judged acceptable for publication (included in the appendix in first annual report – November 2006). With our new monoclonal antibodies derived in Task 2a we are confident we will generate data on the protein levels that will complete the manuscript and make it acceptable for publication. Our results to date from this project were presented as a poster at the DOD Innovative Minds in Prostate Cancer Today meeting held in Atlanta, GA in September 2007 (Appendix).

Conclusions

We have generated data that show that the region of increased copy number in prostate cancer at 18q22.1, originally detected by array CGH, can be minimized to a 680 Kb region that contains the cadherin-7 gene. This increased copy number of the cadherin-7 gene is specific to prostate cancer and is not found in 11 other common cancers. The increased copy number of the cadherin-7 gene also results in increased levels of cadherin-7 mRNA in prostate tumors. We are currently testing new monoclonal antibodies to cadherin-7 to use in immunohistochemistry experiments to determine if the increase in gene copies affects the level of protein. We have performed knockdown experiments of the cadherin-7 mRNA in a prostate cancer cell line and we will be analyzing the cadherin-7 protein levels in these cells. We will subsequently evaluate these cells lines with reduced cadherin-7 for invasive and tumorigenic potential.

We have not found that increased number of the cadherin-7 gene is correlated with the stage or Gleason score of the prostate tumors and may be an early marker of prostate cancer. Since the cadherins are a class of cell adhesion molecules and the type II cadherins are mesenchymal cadherins not normally expressed in epithelial cells, the increased expression of cadherin-7 may be a marker of the tumorigenic phenotype. Assays to detect cadherin-7 in

prostate tissue have the potential to be developed into clinically-useful biomarkers for prostate cancer.

References

Kools P, Van Imschoot G, van Roy F. 2000. Characterization of three novel human cadherin genes (*CDH7*, CDH19, and CDH20) clustered on chromosome 18q22-q23 and with high homology to chicken cadherin-7. Genomics 68:283-295.

Rubin MA, Mucci NR, Figurski J, Fecko A, Pienta KJ, Day ML (2001) E-cadherin expression in prostate cancer: a broad survey using high-density tissue microarray technology. Human Path 32:690-697.

Tomita K, van Bokhoven A, van Leenders GJLH, Ruijter ETG, Jansen CFJ, Bussemakers MJG, Schalken JA (2000) Cadherin switching in human prostate cancer progression. Cancer Res 60:3650-3654.

Appendix
IMPACT 2007 Meeting Abstract

Log Number: PC010614

Title: GAIN OF COPY NUMBER OF AN 18q22.1 REGION THAT INCLUDES THE CADHERIN-7 GENE IN PROSTATE CANCER

Author(s):
Teresa L. Johnson-Pais; Veronica E. Contreras-Shannon; Sapna Vijayakumar (Mount Sinai School of Medicine, New York, New York); Susan L. Naylor and Robin J. Leach.

Presenter: Teresa L. Johnson-Pais

Abstract:
Genomic alterations of the 18q chromosomal region have been observed in prostate cancer. We performed array comparative genomic hybridization experiments using prostate cancer specimens and identified the gain of copy number of a small region at 18q22.1. This relatively gene-poor region includes two cellular adhesion genes, the type II cadherins cadherin 7 and cadherin 19. Alteration of expression of another type II cadherin, cadherin 11, is implicated in tumor invasiveness and metastasis in both breast and prostate cancer. Therefore, we sought to study if the increased copy number of the 18q22.1 region in prostate cancer results in upregulation of expression of the type II cadherins, cadherin 7 and/or cadherin 19, which may play a role in prostate cancer development and/or progression. We verified the gain of copy number of this region using fluorescence in situ hybridization (FISH) and determined the copy number to range from 3-7 copies. The smallest region of copy number alteration was identified by performing FISH on paraffin-embedded prostate tumors using probes derived from bacterial artificial chromosomes containing 18q22.1 genomic sequences. This narrowed region was 680 kilobases and included the cadherin 7 gene and no other known genes. Additionally, FISH analyses of 12 other tumor types showed that the gain of copy number at 18q22.1 was specific to prostate tumor samples. A survey of 40 prostate tumors with various Gleason scores and stages showed that the gain of copy number of this region was not associated with a particular stage or grade. Polyclonal rabbit antibodies generated against small peptides from cadherins 7 and 19 were created for use in immunohistochemistry experiments to analyze the level of protein associated with extra copies of the gene. Extensive testing of these antibodies revealed they were inadequate for immunohistochemistry. Therefore, new antibodies are being generated against bacterially expressed cadherin 7 and cadherin 19 fusion proteins. We have knocked down RNA expression of cadherin 7 using RNA interference in the 22Rv1 prostate cancer cell line. Following the generation of antibodies to detect protein expression in the knocked-down 22Rv1 cells, *in vitro* assays for cellular invasiveness and *in vivo* assays for tumorigenicity will be performed. IMPACT: The discovery of increased copy number of a cellular adhesion gene in prostate cancer could lead to a better understanding of how normal cellular adhesion is altered during acquisition of the metastatic phenotype. Understanding these events could potentially lead to the development of new therapeutics designed to interfere with the metastatic process.